A Research Note

Effects of Temperature and Oxygen on the Growth of *Listeria monocytogenes* at pH 4.5

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ABSTRACT

Growth effects were studied using tryptose phosphate broth adjusted with hydrochloric acid. The microorganism survived for extended periods at low incubation temperatures (5 and 10°C), and grew at intermediate temperatures (19 and 28°C). Aerobic incubation at 37°C resulted in relatively rapid inactivation of the organism; however, when oxygen was restricted the organism recovered and survived for extended periods. Oxygen restriction enhanced the growth rate at 19°C. Results demonstrated temperature and oxygen availability interacted to influence survival of *L. monocytogenes* in low pH environment.

INTRODUCTION

TRADITIONALLY, *Listeria monocytogenes* has been considered relatively sensitive to acid environment, being unable to grow at pH <5.5 (Gray and Killinger, 1966). However, several recent studies indicated that, in microbiological media, the organism could grow at initial pH levels as low as 4.4 (Conner et al., 1986; George et al., 1988; Sorrells et al., 1989; Parrish and Higgins, 1989; Petran and Zottola, 1989; Farber et al., 1989; Buchanan and Phillips, 1990). A factor that appears important in regard to growth of *L. monocytogenes* in a low pH environment is the temperature of incubation. Sorrells et al. (1989), Parrish and Higgins (1989), and Farber et al. (1989) reported incubation temperature had a relatively slight but distinct effect on the minimum pH that would support growth of the microorganism. In a more detailed study George et al. (1988) reported the minimum pH that supported growth of *L. monocytogenes* was 4.4, 4.4, 4.6, 4.6, 5.2 at 30, 20, 10, 7, and 4°C, respectively.

While those studies identified an interaction between incubation temperature and minimum pH, they did not assess the effects on growth kinetics. This information is needed to estimate how rapidly and to what extent the organism would grow under such conditions. Further, those studies were largely performed using aerobic conditions. Recent studies in our laboratory (Buchanan et al., 1989; Buchanan and Phillips, 1990) have suggested the organism may more readily tolerate adverse conditions when grown in an oxygen restricted environment. Accordingly, the objective of our study was to determine the effects and interactions of incubation temperature and atmosphere on growth kinetics of *L. monocytogenes* in microbiological media at pH 4.5. This pH was selected, in part, because it was often used as a target pH for control of other pathogens such as *Salmonella* and *Clostridium botulinum*.

METHODS

Bacterium

Listeria monocytogenes Scott A was used throughout the study. Stock cultures were maintained in Brain Heart Infusion (BHI) Broth at 4°C. Starter cultures were grown aerobically on a rotary shaker (150 rpm) in Tryptose Phosphate Broth (pH 7.2) (TPB) (Difco) for 24 hr at 37°C.

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Culture techniques

The bacterium was cultured aerobically and anaerobically using the technique of Buchanan et al. (1989). Briefly, the TPB was adjusted to pH 4.5 using concentrated HCl, and transferred in 50 ml portions to either 250-ml Erlenmeyer flasks (aerobic cultures) or 250-mL trypsinizing flasks (anaerobic cultures). The flasks were sealed with foam plugs or screw caps + rubber septa, respectively, sterilized by autoclaving, and pre-equlibrated to the desired incubation temperature (5, 10, 19, 28, or 37°C). The flasks were inoculated to a target level of 10^3 cfu/ml. The anaerobic cultures were flushed with N_2 10 min, and sealed. This reduced oxygen content of the flasks to 85–145 ppm. All flasks were then incubated at the appropriate temperature on rotary shakers (150 rpm). At least three replicate cultures were followed for each temperature/atmosphere combination.

A limited number of trypsinizing flask cultures were overlayed with 15 mL of presterilized paraffin oil to enhance anaerobiosis. The oil was added immediately after inoculation. These cultures were then flushed with nitrogen and incubated without agitation.

Periodically, 2.5 mL samples were removed from the Erlenmeyer and trypsinizing flasks using a pipette and a hypodermic needle + syringe, respectively. After diluting appropriately with sterile 0.1% peptone water, samples were plated in duplicate on BHI Agar plates using a Spiral Plater (model D, Spiral Systems, Bethesda, MD). All plates were incubated 24 hr at 37°C and enumerated using a Spiral Systems Colony Counter (model 500A). The pH of the 0-hr samples were determined to ensure maintenance of the target pH.

Growth curves

Where appropriate, growth curves were generated using the Gompertz function as described by Buchanan et al. (1989), and used to calculate generation times (GT), exponential growth rates (EGR), lag phase durations (LPD), and maximum population densities (MPD).

RESULTS

AEROBIC GROWTH of *L. monocytogenes* Scott A in TPB at pH 4.5 was highly dependent on incubation temperature (Fig. 1a). Active growth (i.e., increase in population density > 1 log cycle/mL) was observed in conjunction with incubation temperatures of 19 and 28°C. The higher temperature appeared optimal in regard to growth rate; however, a greater maximum population density was observed with 19°C (Table 1). The microorganism did not grow at 5 and 10°C, but survived essentially unchanged for extended periods. *L. monocytogenes* died off relatively rapidly when the incubation temperature was 37°C.

The effect of incubation temperature on growth of *L. monocytogenes* at pH 4.5 in sealed, nitrogen-flushed flasks was similar to the aerobic cultures in regard to overall pattern of response, though differences associated with oxygen availability were apparent (Fig. 1b). Growth was again observed only at 19 and 28°C, with extended survival occurring at 5 and 10°C. The growth kinetics parameters (Table 1) observed with the aerobic and anaerobic 28°C cultures were similar. However, the 19°C anaerobic cultures had decreased LPD's and

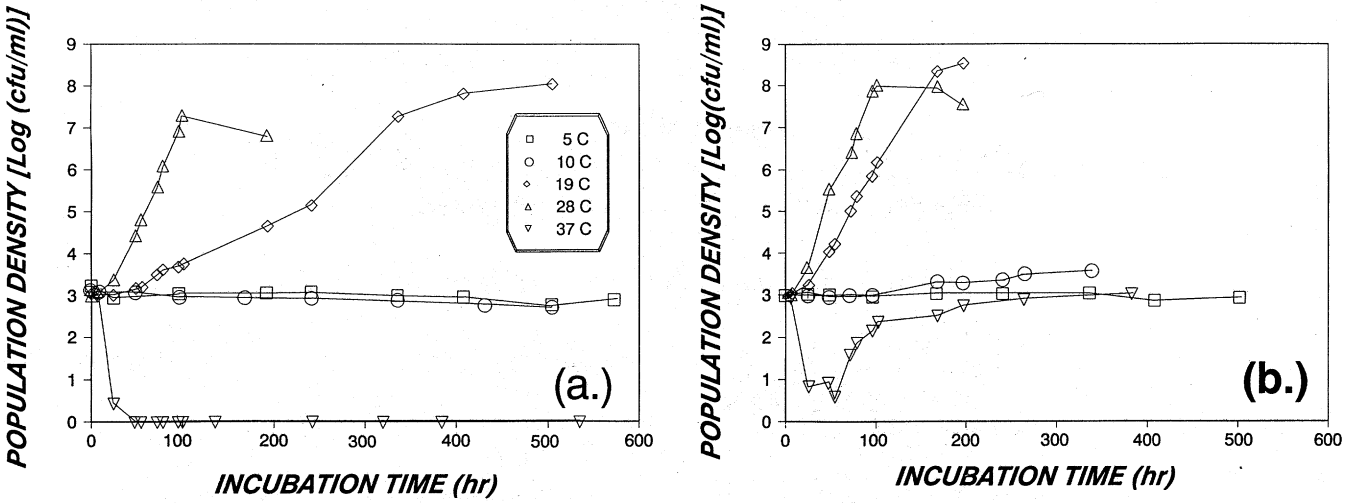

Fig. 1—Effect of incubation temperature on aerobic (a) and anaerobic (b) growth of Listeria monocytogenes *Scott A in tryptose phosphate broth adjusted to pH 4.5. Means of three determinations.*

Table 1—Growth kinetics of cultures that grew in tryptose phosphate broth adjusted to pH 4.5.[a]

Atmosphere	Incubation temp (°C)	Gompertz values A	C	B	M	LPD (hr)	EGR [Log (CFU/mL)/hr]	GT (hr)	MPD Log (CFU/mL)
Aerobic	19	3.0	6.0	0.0072	219.7	80.6	0.016	19.1	9.0
		(0.1)	(0.2)	(0.0004)	(11.6)	(12.6)	(0.001)	(0.4)	(0.2)
	28	3.1	4.1	0.0489	52.4	30.8	0.073	4.1	7.2
		(0.1)	(0.2)	(0.0060)	(3.4)	(1.5)	(0.005)	(0.3)	(0.2)
Anaerobic	19	3.0	6.3	0.0193	79.2	27.3	0.044	6.8	9.2
		(0.1)	(0.2)	(0.0006)	(2.6)	(2.3)	(0.001)	(0.1)	(0.1)
	28	3.0	5.4	0.0376	47.8	21.1	0.074	4.1	8.4
		(0.1)	(0.2)	(0.0031)	(2.1)	(0.9)	(0.007)	(0.4)	(0.2)

[a] Average of three independent determinations; values in parentheses are standard deviations. See text for definitions of abbreviations.

GT's as compared to the aerobic cultures. Again, greater MPD's were observed with the 19°C cultures. The greatest difference between the aerobic and anaerobic cultures was with incubation temperature of 37°C. After an initial decline in population density, two of the three replicate cultures recovered to a level approximating the initial inoculum, remaining at that level for extended periods. The third culture dropped below the limits of detection and did not recover.

The differential response between the aerobic and anaerobic cultures at 37°C was studied further by simultaneously running both aerobic and anaerobic cultures. Included was a third group of cultures consisting of sealed trypsinizing flasks that were overlayed with paraffin oil and incubated without agitation to provide an even more restrictive environment in regard to available oxygen (data not shown). The aerobic cultures again died off. After an initial decline, all of the anaerobic cultures recovered at least to the level of the inoculum. Two of the three cultures overlayed with paraffin oil behaved in a similar manner, while the third did not recover from the initial decline in population density.

DISCUSSION

OUR STUDY extended the recent body of literature indicating that, at least in microbiological media, *L. monocytogenes* could survive and grow at pH 4.5 (George et al., 1988; Sorrells et al., 1989; Ahamad and Marth, 1989; Petran and Zottola, 1989; Parish and Higgins, 1989). The microorganism's response to this low pH was highly dependent on incubation temperature, with survival at low temperatures (5 and 10°C), growth occurring in mid-range (19 and 28°C), and some degree of inactivation at the upper end of the range (37°C). This pattern was similar to that reported by Sorrells et al. (1989); however, a kinetics approach allowed quantitative assessment of the growth at pH 4.5 including the impact of oxygen availability.

Oxygen content had two distinct effects on the microorganism. At 19°C, a growth-permissive temperature, anaerobiosis enhanced growth by decreasing both LPD's and GT's (Table 1). Restricting oxygen content appeared to help the organism overcome the combined adverse effects of nonoptimal pH and temperature since no difference in the kinetics of aerobic and anaerobic cultures was observed at 28°C. The second even more distinct effect was the ability of oxygen to enhance destruction of *L. monocytogenes* when the cells were incubated at 37°C. The initial inactivation was indicative of the combination of low pH and elevated incubation temperature interacting to cause a state of "acid injury" (Buchanan et al., 1988). If oxygen were restricted, in most instances the organism had the ability to repair the physiological lesion(s) associated with acid injury, leading to recovery and subsequent long term survival of the cells. However, in presence of oxygen the lesions were exacerbated, leading to permanent inactivation of the culture. The underlying cause of this oxygen effect was not known. However, it seemed reasonable to hypothesize that one of the physiological lesions resulting from the combination of low pH and elevated temperature was a transient inactivation of enzymes that detoxify active oxygen species such as peroxides and superoxides (Lehninger, 1975). In an anaerobic environment, this would not be a critical lesion, particularly if cell systems could subsequently repair the damage. However, in an aerobic system even the transient inactivation of these enzymes would be fatal as the normal metabolic activity of the cell continued to generate toxic oxygen species. Catalase and superoxide dismutase have been hypothesized to play a role in allowing *L. monocytogenes* to survive adverse intracellular environments (Welch, 1987) and sublethal thermal stress (Dallmier and Martin, 1988).

L. monocytogenes was generally considered to grow optimally at 30–37°C (Rosenow and Marth, 1987; Petran and Zottola, 1989), when other growth factors were optimal. Our results

suggested that *L. monocytogenes* better tolerated adverse conditions when incubation temperatures were somewhat suboptimal. Likewise, though the microorganism was generally considered to grow better aerobically, limiting oxygen appeared to enhance survival and growth when other growth factors were suboptimal. Buchanan and Phillips (1990) also concluded that *L. monocytogenes* was well adapted to microaerophillic environments and that limiting oxygen could enhance its ability to grow under adverse conditions. Identification of the underlying physiological mechanisms responsible for enhanced growth or survivability at suboptimal temperatures and oxygen contents requires future research.

Our study and others (George et al., 1988; Sorrells et al., 1989; Parish and Higgins, 1989; Petran and Zottola, 1989; Buchanan and Phillips, 1990) have indicated that under appropriate conditions *L. monocytogenes* could grow at pH levels as low as 4.4. However, this should be considered a "worst case scenario" in that conditions are optimal for growth and survival of the microorganism. For example, Ryser and Marth (1988) reported that *L. monocytogenes* was unable to grow in cultured or uncultured whey at pH values less than 5.4. Similarly Conner et al. (1986) reported that the organism grew at pH 5.6 but not 4.8 in clarified cabbage juice adjusted with lactic acid. A key factor influencing minimum pH appeared to be the identity of the acid used to alter the pH. Hydrochloric acid, employed in the studies reporting growth of *L. monocytogenes* at pH 4.5, was the least inhibitory to the organism (Sorrells et al., 1989). Organic acids employed as acidulants in foods have been reported to be substantially more detrimental (Sorrells et al., 1989; Ahamad and Marth, 1989; Farber et al., 1989), thereby enhancing control of the bacterium.

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